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REGULAR ARTICLE

Dynamic metabolic profiling in vegetable soybean seed development

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ABSTRACT

The edible quality of vegetable soybean is significantly associated with the number and type of metabolites. In the present study, metabolites in new vegetable soybean variety Mindou 6 at 49 to 67 days after flowering was investigated using gas chromatography coupled with mass spectrometry. The results showed that a total of 102 metabolites were identified from the vegetable soybean seeds, including 36 organic acids (35.3%), 21 amino acids, 11 polyols, 9 sugars, 7 phosphoric acids, 5 amines, 4 fatty acids, 4 nucleotides, and 5 other components. The principal component analysis indicated that the metabolites of amino acids, organic acids and sugars were greatly related to the quality of the vegetable soybean at the filling stages. A total of 40 differential metabolites were identified, including 14 amino acids, 13 organic acids and 2 sugars, and were found to be mainly involved in the metabolism of glycolysis, amino acids and phenylpropanoid. We systematically analyzed the change in the contents of metabolites in the developmental seeds of Mindou 6 and its effects on seed nutrition and eating quality. The suitable picking time was determined by combining the fresh pod yield and appearance quality. Taken together, these results can provide a theoretical reference for vegetable soybean breeding for high quality and efficient production.

Keywords: Gas chromatography coupled with mass spectrometry (GC-MS); Metabolites; Vegetable soybean

INTRODUCTION

The vegetable soybean, called "Edamame" in Japan or "Mao Dou" in China, is harvested at the reproductive stage ranging between R6 and R7 when seeds are immature and pods are not transitioning to a yellow color (Mimura et al., 2007; Czaikoski et al., 2013). This soybean is an excellent source of proteins, free amino acids, carbohydrates, dietary fibers, vitamins, minerals and phytoestrogens (Song et al., 2003), and has developed into a popular market as a large vegetable (Duppong and Hatterman-Valenti, 2013; Zhang et al., 2013). The most obvious features in appearance quality are large pods and seeds, and a green color. As eating quality is an important factor on influencing market share, the standard of eating quality for vegetable soybeans demands a taste that is fragrant, sweet, soft and waxy. Moreover, the most important eating quality for vegetable soybean is that it is sweet and possesses a umami experience in taste. The content of soluble sugar, starch, fat and free amino acid is higher in the vegetable soybean, as evident by the sweeter taste, waxy and soft texture, and a stronger umami (Zhang et al., 2006). The selection of the suitable germplasm of vegetable soybean could provide a good source of nutrition for diet. The chemical variability linked with the eating quality of vegetable soybean, which mainly includes high content of soluble sugar, free amino acids and organic acids. This study focused on the effects of sugar, starch, protein, amino acids on eating quality of vegetable soybean (Zhang et al., 2006; Wang and Wang, 2002), systematic studies on the type and number of metabolites affecting eating quality have rarely been reported.

The metabolite is the final product of gene expression, and the plant phenotype is significantly associated with the number and type of metabolites (Fiehn, 2002). Using metabolism, it is helpful for high throughput to explore the metabolites closely related to plant growth and development. With the emergence of gene expression, metabolites were used to further explain the molecular mechanism of plant growth and development. In recent years, with the development of mass spectrometry and analytical techniques, metabolomics is widely used and has been accepted as an innovative tool (Shepherd et al., 2011; Tohge et al., 2011). A diverse set of antioxidant metabolites,

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including tocopherols, flavonoids, phenylpropanoids, and ascorbate precursors, which are likely responsible, at least in part, for the greater tolerance of vegetable soybeans to high temperatures during seed development, were found to be enriched in seeds of the heat tolerant genotype (Chebrolu et al., 2016). Using metabolomics to study *Brassica napus* and *Brassica campestris*, the results showed that a higher content of polyunsaturated fatty acids and sucrose were observed in turnip rape, while the overall oil content and sinapine levels were higher in the oilseed rape (Kortesniemi et al., 2015).

To date, there are many vegetable soybean varieties bred and cultivated in China. Until the present study was published, there were no comprehensive studies available that reported the dynamic metabolic profiling in vegetable soybean filling seeds. In this study, the metabolites of vegetable soybean seeds with high yield and good quality were analyzed at various time points to assess the metabolic levels in order to improve the quality of vegetable soybeans and provide theoretical guidance.

MATERIALS AND METHODS

Plant material

Vegetable soybean (*Glycine max*) 'Mindou 6' was newly bred by the Institute of Crop Sciences, Fujian Academy of Agriculture Science, and approved by crop variety approval committee of Fujian province in 2013. In autumn 2014, soybean Mindou 6 plants were grown in the experimental field. To investigate the dynamic metabolites profiles of the seeds at the filling stage, the flowering days of 100 plants were tagged. Only the pods of similar size from the same part of the tagged plants were harvested, and a total of 20 pods from 20 different plants were mixed as a sample. Pods including seeds were sampled at 6-day intervals from initiation of seed formation to suitable to eat as vegetable soybean. Pods appeared light yellow at 67 days after flowering (DAF). At the whole seed-filling period (DAF49~67), the color of seeds had no significantly difference. At 49, 55, 61 and 67 DAF, developing pods including seeds were harvested and stored in liquid nitrogen until used. Meanwhile, 100 similarly sized pods with the same sampling day were used to attain the 100-fresh-pod weight.

Metabolite extraction

Seed sample extraction was performed as previously described with slight modification (Lisec et al., 2006). Samples were extracted with methanol, followed by the addition of Ribitol (0.2 mg·mL^{-1} stock in ddH_2O) as an internal quantitative standard. Then chloroform were added, followed by centrifugation at 2,200 × *g* for 10 min to obtain a clear solution. The supernatant was transferred and dried under moderate nitrogen. The dried samples were dissolved in methoxyamine pyridine (15 mg·mL^{-1} solution). Lastly, 60 µL of an MSFTA reagent (containing 1% TMCS) was added and incubated for 60 min at 25 ºC. The tube was then centrifuged for 10 min at 12,000 × *g*. Retention indices were calibrated with the addition of a C8-C20, C21-C40 *n*-alkane mixture to each sample (Sanchez et al., 2012). To avoid the systematic error, all of the samples were randomly sampled and processed at once. All experiments were processes in batches and repeated six times.

GC-MS analysis

The extracted samples were analyzed using an Agilent 7890A GC system coupled to an Agilent 5975C intert XL EI/CI mass spectrometric detector (MSD) system (Agilent Technologies, Santa Clara, CA, USA). Gas chromatography was performed on an HP-5MS capillary column (5% phenyl methyl silox: 30 m × 250 µm i.d., 0.25 µm film thickness, Agilent J & W Scientific, Folsom, CA, USA) to separate the derivatives. One microliter of derivatized sample was injected with a 20:1 split injection ratio. The injection temperature was 280 ºC, the interface was set to 150 ºC and the ion source was adjusted to 250 ºC. The temperature gradient program was as follows: initial temperature set at 80 ºC for 5 min, followed by a 20 ºC·min^{-1} rate increase up to 300 ºC and then maintaining the temperature at 300 ºC for 6 min. The carrier gas (helium) achieved a constant linear velocity at 1 mL·min^{-1}. Mass spectrometry was determined by the full-scan method ranging from 35 to 780 (m/z) at the scan speed of 1,000 u/s. The ionization mode displayed an electron impact at 70 eV and the detector voltage was 0.9 KV.

Metabolites identification and statistical analyses

Peaks with the signal to noise ratio >6 were picked-up by Agilent ChemStation. Raw GC/MS data were converted into CDF format (NetCDF) using Agilent GC/MS 5975 data analysis software and were subsequently processed by the XCMS (www.bioconductor.org) using XdCMS default settings with the following changes (Vanholme et al., 2012): xcmsSet (fwhm=3, snthresh=3, max=300, mzdiff=0.5, step=0.1, steps=2), rector(method="linear", family="gaussian", plottype="mdevden") and bandwidth (bw) of 5. The data were normalized on the basis of the abundance of the internal standard and transformed with unit variance scaling. Identification of metabolites in samples was performed by searching in two databases, namely the National Institute of Standards and Technology (NIST) database and the Golm Metabolome Database (GMD) (Kopka et al., 2005).

Multivariate statistical analyses such as principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were carried out using Soft Independent Modeling of Class Analogy (SIMCA)-P (version 11.0,

Umetrics AB, Umea, Sweden). Any notable differences between the metabolites were screened by the loading plot in PLS-DA. The variables with VIP (Variable Importance in the Projection) values greater than 0.8, which played significant roles in the classification, were selected for further analysis. Subsequently, independent *t*-test was used for excluding the variables that were not significantly different ($P > 0.05$).

RESULTS

Phenotype of pod and seed in different periods

After entering the reproductive growth stage, the pod length and width of the vegetable soybean develop first, and then the size and fresh weigh of seeds begins to develop until its pod length and width arrive to its inherent size. The fresh 100-pod weight of Mindou 6 was 121.30, 181.24, 221.86 and 230.12 g at four different stages, respectively, which showed that the fresh pod weight increased gradually with a tendency of fast first and then slow. Compared to DAF49, fresh pods weighed at at DAF55, DAF61 and DAF67 were 49.41%, 82.90% and 89.71% higher, respectively. Pods appeared light yellow at DAF67. At the whole seed-filling period (DAF49-DAF67), the color of the seeds was not significantly different. Taken together, the suitable period to pick Mindou 6 was found to be between DAF61 and DAF67, and the harvest must be completed before DAF67.

Metabolites changes during seed development

Three hundred and fifteen peaks were detected, 102 of which could be specifically identified by the NIST and GMD library and used for further statistical analysis. These metabolites could be nine categories of chemicals, including organic acid, amino acid, polyol, sugar, phosphoric acid, amine, fatty acid, nucleotide and other. Thirty-six species in organic acids accounted for 35.3%, followed by 21 kinds of amino acids accounting for 20.6%, 11 kinds of polyols accounted for 10.8%, 9 sugars for 8.8%, 7 phosphoric acids for 6.9%, 5 amines for 4.9%, 4 kinds of fatty acids and nucleotides respectively, each accounted for 3.9%, and 5 other substances accounted for 4.9%.

Principal component analysis (PCA) was used to visualize the developmental patterns of the seeds and changes in concentrations, with a total of 102 metabolites, of the vegetable Mindou 6 seeds. Four different stages were used for analysis and six repeats, with exception to the samples at DAF49 due to an abnormal repeat deletion, were implemented. The PCA model revealed a statistically significant ($P<0.05$) separation of the samples into four groups (Fig. 1). The metabolites profiles at DAF49 and DAF55 were more closely related than those at DAF61 and DAF67, suggesting that a large portion of new metabolites were synthesized after DAF61. The score plot of PCA for the first two components (R2X =0.810, Q2 0.485) showed a separate trend for the vegetable soybean seeds at the developmental stages (Fig. 1). The first two principal components (PCs) explained 52.5% of the total variance, which PC1 and PC2 accounted for 38.1% and 14.4%, respectively. The metabolites in vegetable soybean seeds which contributed to PC1 was dominated by amino acids (e.g. asparagine, homoserine, threonine, alanine, serine, isoleucine, leucine, valine, proline and lysine) and organic acid (e.g. tetronic acid, suberyl glycine, 2,3-dihydroxybutanedioic acid, succinic acid, malic acid and fumaric acid), while glucose, sorbitol, fructose, melibiose, isomaltose and sucrose were the main contributors of PC2 (Fig. 2, File S1). The resulting composition analysis suggests that the amino acids,

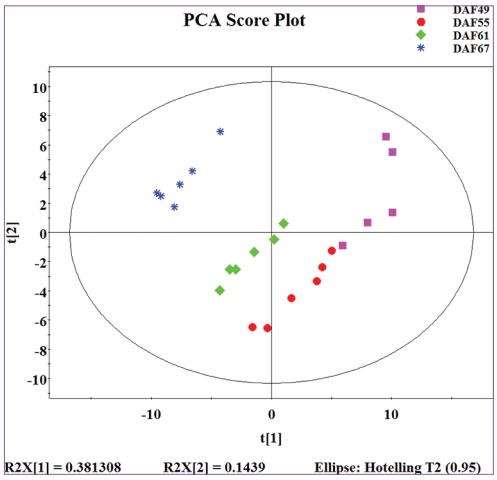

Fig 1. Score of principal component analysis (PCA) of metabolic profiles of vegetable soybean seeds at different stages. Pink squares, red dots, green diamonds and blue asterisks indicate the samples at DAF49, DAF55, DAF61 and DAF67, respectively. The first two principal components (PCs) explained 52.5% of the total variance, which PC1 and PC2 accounting for 38.1% and 14.4%, respectively.

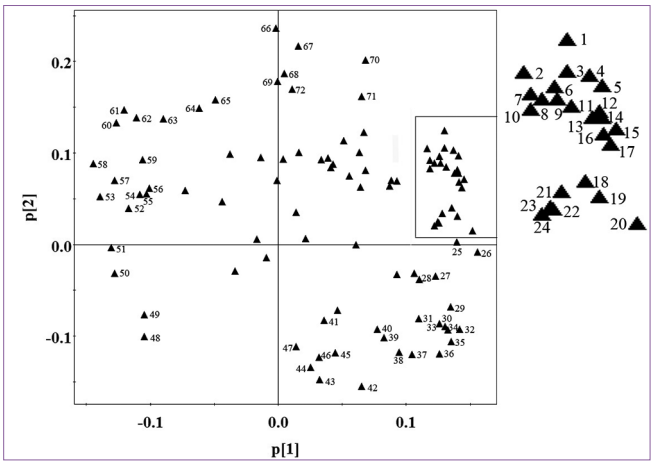

Fig 2. Loading plot of PCA of metabolic profiles of vegetable soybean seeds at different stages. The metabolite numbers are shown in File S1.

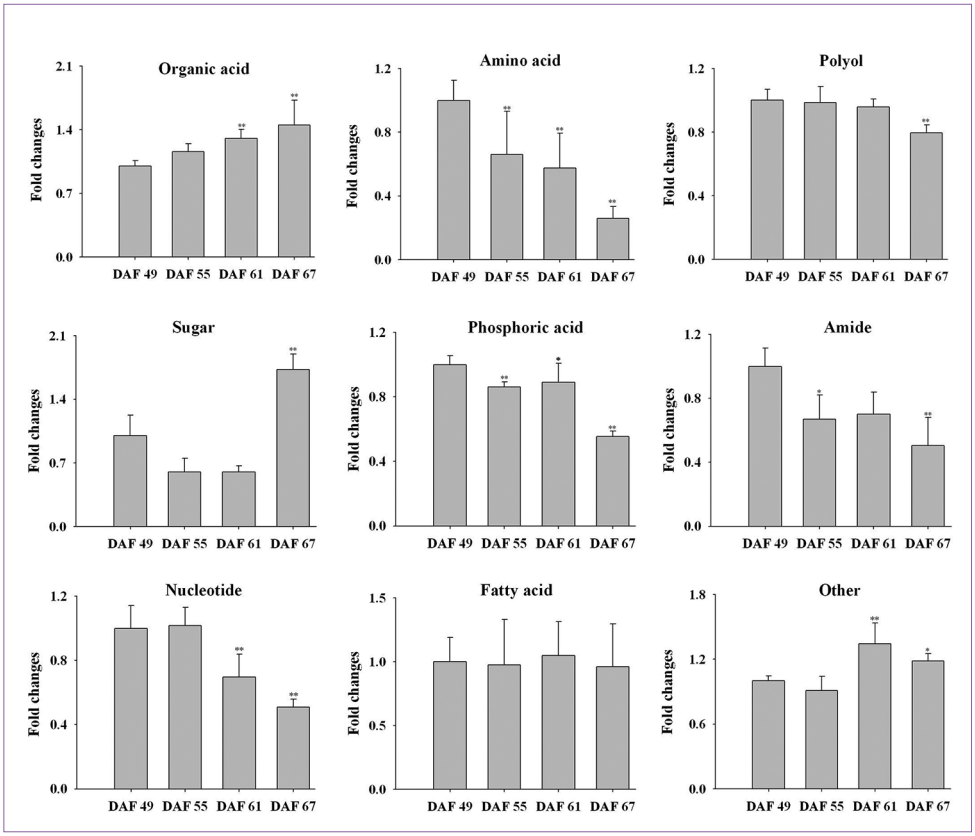

Fig 3. Fold changes in metabolites of vegetable soybean seeds at four developmental stages. Data are presented as mean ± standard deviation. DAF, days after flowering. Asterisks indicate statistically significant differences compared to DAF 49 (* P <0.05; ** P <0.01).

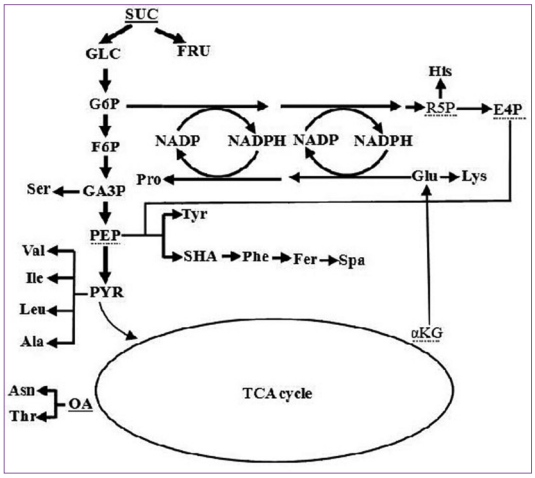

Fig 4. The proposed metabolic pathway of vegetable soybean seeds at different stages. The metabolites with solid lines represent a detectable metabolite; however, the difference is not significant. Dotted lines represent the non-detected metabolites. SUC: sucrose; GLC: glucose; FRU: fructose; G6P: glucose-6-phosphate; F6P: frucose-6-phosphate; GA3P: glyceric acid-3-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvic acid; OA: oxalic acid; αKG: α-ketoglutaric acid; R5P: ribose-5-phosphate; E4P: erythrose-4-phosphate; Ser: serine; Val: valine; Ile: isoleucine; Leu: leucine; Ala: alanine; Asn: asparagine; Thr: threonine; His: histidine; Pro: proline; Glu: glutamic acid; Lys: lysine; Tyr: tyrosine; SHA: shikimic acid; Phe: phenylalanine; Fer: ferulic acid; Spa: sinapinic acid.

organic acids and carbohydrate metabolites have a great relationship with the quality of vegetable soybean during development.

Further analysis indicated that the content of organic acid, sugar and other metabolites increased gradually with seed development. On the contrary, that of amino acid, phosphoric acid, amine and nucleotide metabolites gradually decreased, and minimum changes was noted in the content of polyol and fatty acids (Fig. 3). Among these nine categories, organic acid at DAF67 exhibited the highest content. However, amino acid content exhibited the highest content at DAF49 and then decreased slowly at DAF55 to DAF61, and decreased rapidly from DAF61 and reached the lowest content at DAF67. In contrast, the levels of sugars were decreased from DAF49 to DAF55 and stayed relatively stable from DAF55 to DAF61. However, the sugar levels dramatically increased again at DAF67, mainly due to a sharp rise in sucrose at DAF67 which the level of sugar shapely increased from 193.01 at DAF61 to 1801.72 at DAF67 (the data was not shown). With the development of seeds, the levels of fructose and glucose first decreased and then increased, and the fructose content was about 6.21 – 7.68 times that of glucose (Table 1).

Table 1: Different metabolites isolated from developing seeds

Category	Metabolite	VIP	Content in different stage			
			DAF 49	DAF 55	DAF 61	DAF 67`
Amine	2,5-Diaminovalerolactam	0.864975	6.91±3.20	3.08±2.38	1.51±0.88	1.54±0.87
	N-Acetyl glucosamine	0.969397	1.16±0.42	0.52±0.12	0.36±0.25	0.38±0.06
Amino acid	Alanine	0.817621	38.04±8.02	19.28±11.72	17.05±14.01	5.58±3.52
	Asparagine	0.917946	2554.20±329.05	1860.33±651.81	893.07±351.34	228.96±127.11
	Glutamic acid	1.51271	307.21±153.82	65.65±25.08	322.78±144.63	76.19±26.88
	Histidine	0.905464	699.10±358.08	219.20±253.12	33.45±40.85	7.78±11.91
	Homoserine	0.969544	15.91±1.16	9.37±3.47	3.33±1.69	1.41±0.58
	Isoleucine	0.949397	108.46±28.60	42.30±24.93	39.53±24.55	23.02±11.19
	Leucine	0.833701	108.38±36.25	49.90±34.78	34.51±25.80	19.69±11.67
	Lysine	0.878405	137.13±62.00	50.80±40.60	44.61±36.94	14.51±11.45
	Phenylalanine	1.09651	33.40±4.47	20.02±5.70	25.80±7.51	17.71±4.54
	Proline	0.93897	165.27±63.84	52.59±36.68	38.59±31.42	16.62±10.18
	Serine	0.801906	511.64±107.70	253.37±172.53	221.26±203.09	74.26±51.73
	Threonine	0.839582	78.78±11.15	39.92±20.94	32.39±24.93	15.81±9.45
	Tyrosine	1.07158	101.86±19.16	43.52±15.13	39.48±15.46	38.93±9.65
	Valine	0.981429	239.54±76.69	81.62±46.34	58.72±31.11	34.04±15.87
Organic acid	2,3-Dihydroxybutanedioic acid	1.01876	14.73±1.13	8.97±0.41	8.31±1.12	5.30±0.35
	2-Aminoadipic acid	1.51819	24.16±2.33	34.40±7.85	44.43±8.30	15.72±3.57
	4-Hydroxy-3-methoxybenzoic acid	1.03397	6.50±0.87	10.18±0.47	12.21±1.97	12.06±0.93
	Ferulic acid	1.01574	9.26±1.12	12.35±0.52	16.29±2.67	16.22±1.98
	Gluconic acid	1.1915	11.93±1.17	20.91±2.88	17.50±3.94	20.58±2.89
	Glyceric acid	1.41088	43.21±4.83	54.70±2.83	41.23±8.90	44.25±4.11
	Nicotinic acid	1.0461	1.78±0.19	2.27±0.38	2.16±0.32	3.24±0.33
	Pyruvic acid	0.832662	22.56±10.79	12.03±3.13	13.72±4.07	10.20±1.51
	Shikimic acid	1.03754	40.69±3.01	35.12±2.13	25.98±4.36	24.49±2.50
	Sinapinic acid	1.55069	18.06±1.38	12.87±0.89	19.58±3.27	18.10±2.01
	Suberyl glycine	0.881479	17.81±6.66	9.06±6.11	2.56±1.11	0.98±0.37
	Succinic acid	0.916328	105.28±5.58	83.51±5.14	79.00±7.30	70.97±8.77
	Tetronic acid	1.01197	31.76±2.49	28.47±0.99	18.56±2.36	9.37±1.54
Others	Catechine	0.853393	3.18±0.66	2.19±0.54	1.76±0.33	1.77±0.54
Sugar	Fructose	1.34233	1280.18±309.92	572.87±44.46	583.52±61.67	885.12±73.78
	Glucose	1.29197	133.76±48.83	75.24±7.41	75.90±13.83	142.57±20.70
Polyol	Galactosylglycerol	1.53055	18.05±1.24	8.99±0.73	11.31±1.53	16.29±1.84
	Sorbitol	1.22831	286.38±185.62	93.69±7.52	100.51±24.76	258.18±16.60
	Threitol	1.22614	15.69±2.25	6.34±0.53	7.97±2.03	4.26±0.27
Phosphoric acid	Glucose-6-phosphate	1.49567	5.09±0.84	2.83±0.23	4.03±0.55	4.37±0.29
	Fructose-6-phosphate	1.1456	7.35±6.23	1.44±0.18	4.74±1.62	6.46±1.11
	Glyceric acid-3-phosphate	1.66587	6.07±0.47	2.64±0.42	10.36±2.33	8.09±1.82
	Glycerol-3-phosphate	1.02165	25.59±2.57	19.82±1.95	14.88±2.57	15.39±1.76
	Phosphoric acid	1.10778	3804.99±215.58	3288.56±122.31	3384.19±444.48	2075.88±130.26

Note: VIP is variable influence on projection values. Data are presented as mean±standard deviation.

Differential metabolites during seed development

According to the analysis of one-dimensional variance during metabolites of seed development (FDR $P<0.05$) and VIP >0.8, 40 metabolites were identified from a total 102 metabolites, including 14 amino acids, 13 organic acids, 2 sugars, 3 polyols and 5 phosphoric acids, which exhibited differential changes during seed development (Table 1). To show the regulated pattern of every key metabolic pathway, such as the glycolytic pathway, the metabolism of amino acids and the phenylpropanoid pathway, the metabolism was incorporated into a propositional metabolic pathway (Fig. 4).

As shown in Fig. 4, sucrose, as the precursor, was at a higher level at DAF 67 during seed-filling stage when compared with the other stages but the difference was not significant. However, glucose and fructose derived from the hydrolysis of sucrose were all more abundant. The glucose-6-phosphate precursor presented in the two branches, which was glycolysis and the pentose phosphate pathway. On the one hand, glucose 6- phosphate in glycolysis entered into the pentose phosphate pathway and produced histidine. On the other hand, glutamic acid was produced by α- ketoglutaric acid in the TCA cycle while entering into the pentose phosphate pathway and was further converted

to lysine and proline. Glycerol-3-phosphate produced in glycolysis was converted to serine. One way in which the phenylpropanoid pathway generated tyrosine was through phosphoenolpyruvate in glycolysis, while the other portion of the pathway produced shikimic acid, phenylalanine, ferulic acid and sinapic acid. On one hand, pyruvic acid was the substrate needed to enter into amino acid metabolism pathway to produce valine, isoleucine, leucine and alanine. On the other hand, pyruvic acid acted as an intermediate product of amino acid metabolism by entering into TCA cycle to produce asparagine, threonine and glutamic acid.

DISCUSSION

Vegetable soybean, an environmentally-friendly food, has already been a secure, sanitary, nutritional and health-care food for modern consumers. As an export of vegetable soybean, in addition to yield traits, appearance quality is also a very important commodity (Zhang et al., 2007). As for appearance quality, despite the seeds that maintained a green color, the vegetable soybean pods appeared light yellow at DAF67, which did not meet the remarket demand of pods and seeds staying green (Han et al., 2003; Zhao et al., 2008). In order to obtain higher benefits to ensure higher yield and enhance selling potential, Mindou 6 soybeans were harvested between DAF61 and DAF67.

Such an organoleptic quality of vegetable soybean has been shown to be dependent on various factors, such as different varieties, the harvest stage, the duration between the harvest and processing data, and storage conditions (Czaikoski et al., 2013; Li et al., 2012a; Mozzoni et al., 2009; Song et al., 2013). Soluble sugar content in seeds is an important factor on eating quality (Zhang et al., 2015). Li et al (2012a) reported that sucrose was the highest content and accounted for 70% of soluble sugar at the edible stage of vegetable soybean. It was significantly and positively correlation between sucrose content and sweet taste scores (Wang and Wang, 2002). Meanwhile, the sucrose content was higher in fruit, fresh corn and vegetable soybean, so the effect of sucrose on the edible quality of fruits and vegetables has become the focus of recent research (Li et al., 2012a; Li et al., 2012b; Cao et al., 2011). Among the nine sugars identified in Mindou 6 seeds, the tendency of glucose, fructose and sucrose was similar to that of total sugar, which decreased first and then increased gradually, especially at DAF61– DAF67, the period of rapid increase, when the highest sucrose content was reached. At the whole seed-filling stage, fructose content was about 6-10 times that of glucose. The sweetness of sucrose to the relative sweetness of carbohydrate is 1, while fructose and glucose are 1.2–1.7 and 0.7–0.8, respectively (Bowers, 1992), namely compared to sucrose. Although the sucrose content was higher when compared with fructose, and fructose was higher when compared to sucrose in terms of sweetness, the results suggest that sweet property of vegetable soybean was highly related to the content of sucrose and fructose.

The amino acid content is an important factor, which mainly affects the eating quality aspect of umami of vegetable soybean (Silva et al., 2012; Zoldan et al., 2014). Masuda (1991) showed that the content of starch, sucrose, fructose, glutamic acid and alanine in vegetable soybean was higher, therefore, the flavor was enhanced. A lactic acid bacteria co-culture from paocai brine could significantly increase glutamic acid (umami), sucrose (sweetness), glycine (sweetness), lactic acid (sourness), and γ-aminobutyric acid in PB-paocai, which would endow it with important flavor features (Zhao et al., 2016). Of 21 kinds of amino acids identified from developing seeds, differential amino acids including alanine, glutamic acid, asparagine and 13 other types of amino acids played an important role in seed development and influenced eating quality. Studies have shown that asparagine, alanine and glutamic acid content of vegetable soybean have an important role in determining the quality of the vegetable soybean (Masuda, 1991; Zhao et al., 2016), which is consist with the results of this study. As the seeds matured, the amino acid content gradually decreased, and reached its lowest content at DAF67, which was 54.94% lower than the amino acid levels displayed at DAF61.

As a product of plant primary metabolism and intermediates, organic acid not only regulated plant development, and responded to resistance to nutrient deprivation, metal stress, rhizosphere soil- plant microbe interaction, but organic acid was also one of the most important factors affecting fruit flavor and sensory quality (Wen et al., 2014; Liu et al., 2016; López-Bucio et al., 2001; Mattila and Hellstrom, 2007). Among 36 organic acids, citric acid, succinic acid, fumaric acid, malic acid and oxaloacetic acid were not only intermediate products belonging to the TCA cycle, but were also the substrate capable of producing the most amino acids. Relatively, the highest content of organic acid was citric acid, followed by malic acid and fumaric acid, which was consistent with the report of Song et al (2013). In addition, differential phenolic acids containing shikimic acid, ferulic acid and sinapic acid and other acids were identified by GC-MS, and played an important role in the flavor of vegetable soybean, which may be related to abundant secondary metabolites contained by soybean (Wu et al., 2008). Organic acid content in vegetable soybean seeds gradually increased and reached a climax at DAF67. Although our study analyzed the organic acid content of vegetable soybeans, the effect of organic acid content and composition on the flavor formation of vegetable soybean warrants further investigation.

Polyol, also known as sugar alcohol, mainly acts as nutritional supplements in the food industry, and has certain physiological activity. Therefore, it is used to add sweetness and softness in sugar-free and diet food (Yang and Yang, 2003; Czaikoski et al., 2013). Polyol also affects eating quality of vegetable soybean, however, the content of polyols had no significant difference in the process of seed development, speculating it had little effect on the picking period.

Based on fresh pod weight, appearance quality and change in content of metabolites, the results showed that the fresh pod yield at the latter of the picking period had minimum change, however, the appearance quality worsened with as the picking period was further delayed. During DAF61- DAF67, the amino acid content decreased rapidly, on the contrary, sugar content dramatically increased and organic acid content gradually increased. Great consideration and achievement of marketing factors, such as yield, appearance quality, nutritional quality, eating quality, are necessary to ensure the highest economic benefit. As much, the harvesting of Mindou 6 beans should be completed during DAF61- DAF67, and the last date to harvest should be set to DAF67. Our results show that it is beneficial to sustain a longer pod-picking period at R6~R7 to satisfy the market demand. Additional studies are necessary to determine the process by which vegetable soybean pod-picking can be extended, improvements can be exhibited regarding the expression of seed metabolites in the picking period, as well as the expression of metabolites in different cultivars.

CONCLUSION

During the seed-filling period of vegetable soybean Mindou 6, amino acid, organic acid and sugar metabolites had a significant impact on its quality. Amino acids are known to contribute to the increase in nutrition and umami, while organic acids have been shown to improve its flavor, and sugar is known to increase its sweetness. With regard to yield, appearance quality, nutrition quality, eating quality and other factors, our studies found that the preferred pod-picking period was at DAF61-DAF67 and that the harvest must be completed before DAF67.

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AUTHOR CONTRIBUTIONS

L.G.Q.: Design and supervision of experiment and writing and reviewing the manuscript. Z.Y.M.: Collection of data and writing. H.R.F.: GC-MS work and statistical analysis of data. C.Y.H.: Field experiments.

SUPPORTING INFORMATION

File S1. The metabolite numbers were shown in File S1.

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FILE S1. THE METABOLITE NUMBERS SHOWN IN FIG.2

No.	Metabolite	No.	Metabolite	No.	Metabolite
1	Tyrosine	25	2,3-Dihydroxybutanedioic acid	49	Maltitol
2	N-Acetyl glucosamine	26	Asparagine	50	4-Hydroxy-3-methoxybenzoic acid
3	Lysine	27	Shikimic acid	51	Ferulic acid
4	Valine	28	Cysteine	52	Ribonic acid
5	Isoleucine	29	Uracil	53	3-Deoxy-arabino-hexaric acid
6	Glutamine	30	Phosphoric acid	54	Glucopyranose
7	Phenylalanine	31	Pyroglutamic acid	55	Monomethylphosphate
8	beta-Alanine	32	Tetronic acid	56	Cellobiose
9	Catechine	33	Adenine	57	Nicotinic acid
10	Ethanolamine	34	Fumaric acid	58	Aconitic acid
11	2,5-Diaminovalerolactam	35	Malic acid	59	Citric acid
12	Histidine	36	Malonic acid	60	Glucaric acid
13	Proline	37	myo-Inositol	61	Galactinol
14	Threonine	38	Threonic acid	62	Galactonic acid
15	Leucine	39	Glycolic acid	63	Digalactosylglycerol
16	Serine	40	myo-Inositol-1-phosphate	64	Sucrose
17	Alanine	41	Benzoic acid	65	Isomaltose
18	Succinic acid	42	Uridine	66	Glucose
19	Suberyl glycine	43	Lactic acid	67	Sorbitol
20	Homoserine	44	4-hydroxy-Butyric acid	68	Galactosylglycerol
21	Threonic acid-1,4-lactone	45	2-Aminoadipic acid	69	Fructose-6-phosphate
22	4-Aminobutyric acid	46	2-Hydroxyglutaric acid	70	Fructose
23	Threitol	47	Glyceric acid	71	Spermidine
24	Glycerol-3-phosphate	48	Gluconic acid	72	Glucose-6-phosphate